BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

NOVEL TYPE III SECRETION PATHWAY IN *AEROMONAS SALMONICIDA*, AND USES THEREFOR

FIELD OF THE INVENTION

This invention relates to bacterial secretion systems, and in particular to a newly identified and characterized type III secretion system in *Aeromonas salmonicida*. The invention also encompasses the use of components of the novel secretion system in immunoprotection against *A. salmonicida* infection, as well as other diagnostic and therapeutic uses thereof.

BACKGROUND OF THE INVENTION

Various publications are referenced throughout this publication, and full citations for each of these publications are provided at the end of the Detailed Description.

Aeromonas salmonicida, a Gram-negative, facultatively anaerobic, non-motile, rod shaped bacterium, growing at temperatures around 20°C, is the etiological agent of furunculosis in salmonids, causing most severe economic losses in production farms of salmon and trout. The disease is characterised in the sub-acute or chronic form by the presence of haemorrhagic necrotic lesions in the gills, gut and muscle, while in the acute form fish die apparently from toxaemia without showing particular external signs.

Due to the high contagiousity of the disease and the high mortality in salmon of all ages, particularly in the sea water growers, large amounts of antibiotics are used in closed and open waters for therapy of furunculosis (Munro and Hastings, 1993). Vaccination has become an important strategy to control furunculosis in fish farms (Ellis, 1997). However, the currently applied whole cell antigen vaccines seem to show considerable variability in efficacy, the origin of which remains currently unexplained (Thornton et al., 1993).

Knowledge of the mechanisms of pathogenicity of *A. salmonicida*, and in particular of the main virulence factors involved, is essential in the development of efficient strategies to prevent outbreaks of furunculosis caused by *A. salmonicida*. Currently, several potential virulence factors of *A. salmonicida* have been reported, including a surface-layer protein (Chu et al., 1991), the hemolysins ASH1, ASH3, ASH4 (Hirono and Aoki, 1993), salmolysin (Titball

and Munn, 1985), the serine protease AspA (Whitby et al., 1992) and the glycerolipid-cholesterol acyltransferase (GCAT) (Lee and Ellis, 1990), but their role in pathogenesis is unclear and many of them seem not to play a primary role in virulence. This was demonstrated by *A. salmonicida* strains with deletion mutants of the GCAT and *aspA* genes which had no influence on virulence of the strains in inducing furunculosis.

SUMMARY OF THE INVENTION

A new ADP-ribosylating toxin named AexT (*Aeromonas* exoenzyme T) encoded by the gene *aexT* was identified in a virulent strain of *A. salmonicida*. *A. salmonicida* strains that were propagated for several passages on culture medium had lost expression of AexT, but still retained the *aexT* gene. AexT shows amino acid sequence similarity to the ADP-ribosyltransferase toxins ExoS and ExoT of *Pseudomonas aeruginosa* which are secreted by a type III-dependent secretion mechanism (Yahr et al., 1996). Regulation of *aexT* was shown to be dependent on contact with fish cells and could also be induced by Ca^{2+} depletion of the medium. The *aexT* gene was found to be preceded by a consensus sequence for binding of a transcriptional activator known in *P. aeruginosa* as ExsA which is involved in type III mediated gene expression (Frank, 1997).

Based on these observations, we used broad range gene probes to identify in *A. salmonicida* a novel type III secretion system by means of the gene *acrD* (*Aeromonas calcium response* D) encoding a transmembrane spanning protein. The *acrD* gene has a high similarity to *lcrD*, a protein of the *Yersinia* sp. which is an inner membrane protein of the type III secretion apparatus in *Yersinia* sp. The *acrD* gene is flanked by further typical type III secretion genes which were designated *acr1*, *acr2*, *acr3*, *acr4*, *acrD*, *acrR*, *acrG*, *acrV*, and *acrH*, and which show significant similarity to *pcr1*, *pcr2*, *pcr3*, *pcr4*, *pcrD*, *pcrR*, *pcrG*, *pcrV*, and *pcrH* of *Pseudomonas aeruginosa* and to *tyeA*, *sycN*, *yscX*, *yscY*, *lcrD*, *lcrR*, *lcrG*, *lcrV*, and *lcrH* of *Yersinia enterocolitica*. All these genes play a predominant role in building up the type III secretion apparatus in the respective bacterium, including the regulation of the low calcium response (LCR) and chaperon functions. The genes isolated from *A. salmonicida* belong to the analogue of the *virA* operon, which is central in the type III secretion pathway of many Gram-negative pathogens of human, animals and plants (Fenselau et al., 1992; Gough et al., 1992; Michiels and Cornelis, 1991).

We have also determined that the type III secretion system in *A. salmonicida* is located on a 84 kb plasmid which is rapidly lost upon growth in culture medium. Biosynthesis of AcrV in *A. salmonicida*, the analogue to LcrV in *Yersinia*, requires as a trigger either low Ca^{2+} conditions or contact with fish cells. Upon infection with *A. salmonicida* expressing AcrV, the cultured cells undergo significant morphological changes. Cultures derived from originally

virulent *A. salmonicida* strains, which had lost the type III secretion genes including AcrV, lost virulence as they did not affect rainbow trout gonad cells morphologically after infection. Concomitantly to loss of the type III secretion genes, these cultures lost the expression of the *aexT* gene which specifies the ADP-ribosylating toxin of *A. salmonicida*.

Rainbow trout gonad cells infected with the virulent *A. salmonicida* and incubated in antiserum directed against recombinant AcrV-His protein could be protected from the toxic effect and showed only weak morphological changes. AcrV, which belongs to the type III secretion proteins is a determinative factor involved in virulence mechanisms of *A. salmonicida*, and is expected to provide new insights into basic mechanisms of pathogenicity of bacterial species. The components of the type III secretion system of *A. salmonicida* may be used as antigens for the development of sub-unit vaccines against infection of fish by *A. salmonicida*.

In one embodiment, the invention comprises an isolated 5.7 kb nucleic acid segment (SEQ ID NO:10) containing the type III secretion genes of *A. salmonicida* . In another embodiment, the invention comprises a nucleic acid segment that encodes protein having the amino acid sequence of SEQ ID NOS:1, 2 , 3, 4, 5, 6, 7, 8, and 9, including variants that retain either biological activity or immunogenicity or both. Due to the degeneracy of the genetic code and the possible presence of flanking nucleic acid fragments outside of the coding regions, it will be understood that many different nucleic acid sequences may encode the amino acid sequence of SEQ ID NO NOS:1, 2 , 3, 4, 5, 6, 7, 8, or 9, and variants, and that all such sequences would be encompassed within the scope of the present invention.

In a further embodiment, the invention relates to the use of AcrV as an immunogen, and to the use of AcrV in a recombinant or traditional vaccine to reduce the incidence of infection by *A. salmonicida*.

In another embodiment, the invention provides a means of diagnosing *A. salmonicida*, or other bacteria found to contain AcrV homologues, by the detection of the AcrV protein or the homologous proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a genetic map of the type III secretion genes found in *A. salmonicida*. Boxes with arrowheads indicate open reading frames (ORFs). The size of the different genes (in kilobases) is shown by the scale bar. A restriction map containing restriction enzymes *Sac*I, *Pst*I, *Not*I, *Bam*HI, and *Sal*I is shown. Abbreviation used: acr, *Aeromonas* calcium response.

FIG. 2 is a segregation curve of *A. salmonicida* JF2267. An *A. salmonicida* JF2267 LB-culture was first incubated 2 ½ hrs at 19°C and then at 22°C for 7 hrs. Colony-blotting was performed to analyze the LB-culture at 10 different time points for positive, respectively negative colonies.

FIG. 3 shows a pulsed-field gel electrophoresis of *A. salmonicida* strain JF2267, and strain JF2397. (Lane 1) JF2267, undigested. (Lane 2) JF2397, undigested. (Lane 3) JF2267 digested with *Not*I. (Lane 4) JF2397 digested with *Not*I. (Lane 5) Low Range PFG Marker (New England Biolabs). The white arrows indicate the bands that hybridized on Southern blots with the *acrD* gene probe.

FIG. 4 shows infection of fish cells with *A. salmonicida* ATCC 33658^T, JF2267, and JF2397. RTG-2 cells infected with JF2267 (A), ATCC 33658^T (B), JF2397 (C), and pure PBS (D). RTG-2 cells infected with JF2267 and monospecific polyclonal antibodies against AcrV were protected (E), whereas RTG-2 cells infected with JF2267 and anti-AcrV preserum were not. Pictures were taken 24 hrs after infection, respectively 21 hrs after the protection assay under a phase contrast microscope.

FIG. 5 shows low Ca^{2+} response induced AcrV expression in *A. salmonicida* JF2267. The picture shows an immunoblot reacted with specific rabbit anti-AcrV antiserum. Strains ATCC 33658^T (lane 2), JF2267 (lane 3) and JF2397 (lane 4) were grown in Ca^{2+} depleated medium, harvested by centrifugation and analyzed on 15% SDS PAGE followed by immunoblotting. Lane 1 contains purified recombinant AcrV-His protein as a control.

DETAILED DESCRIPTION

A 5.7 kb segment containing type III secretion genes of *A. salmonicida* that were cloned and sequenced correspond to the *pcr* locus (*Pseudomonas calcium response*) of *Pseudomonas aeruginosa* (Frank, 1997; Yahr et al., 1997b) and the *virA* operon and genes of the following operon of *Yersinia enterocolitica* (Cheng and Schneewind, 2000; Iriarte and Cornelis, 1999; Plano et al., 1991; Skrzypek and Straley, 1993; Motin et al., 1994; Price and Straley, 1989) and other Gram-negative animal and plant pathogens (Fenselau et al., 1992; Gough et al., 1992; Michiels and Cornelis, 1991). The most conserved gene at this locus was revealed to be the *acrD* gene encoding the AcrD protein, which showed 82% identical aa to the transmembrane spanning core proteins LcrD of the injectisome of the *Y. enterocolitica* type III secretion apparatus and PcrD of the injectisome of the *P. aeruginosa* type III secretion apparatus (Yahr et al., 1997b; Plano et al., 1991). Due to this high similarity, we conclude AcrD to have the analogous functions in the injectisome of the *A. salmonicida* type III secretion pathway.

The least conserved protein encoded on the cloned and analyzed segment is AcrV, which shows only 35% identical aa to PcrV of *P. aeruginosa* and 37% identity to LcrV of *Y. enterocolitica*. The main role of LcrV and PcrV, and accordingly also of AcrV, is assumed to be involved in sensing the bacterium-host interactions (Sawa et al., 1999; Bergman et al., 1991). We therefore interpret the significantly higher dissimilarity between AcrV and LcrV or PcrV, compared to the other gene products of the type III secretion locus (Table 2), to be due to the host specificity which seems to be determined by AcrV, LcrV or PcrV.

Our analyses revealed the *A. salmonicida* type III secretion genes to be located on a plasmid of 84 kb. The plasmid was shown to be lost very easily in standard growth media, in particular after a slight raise in growth temperature. Concomitant to the loss of the type III genes in *A. salmonicida*, we detected the loss in virulence of the strain as measured by the infection of RTG-2 fish cell cultures, as well as the loss of production of ADP-ribosylating toxin *aexT* in supernatants and bacterial cell pellets of low Ca^{2+} response induced *A. salmonicida* cultures. It is also noted that AexT biosynthesis induced by contact of *A. salmonicida* with RTG-2 fish cells disappeared in those strains or subcultures that had lost the type III secretion genes. Expression of the *aexT* gene must therefore be regulated by a mechanism which is dependent on type III secretion genes. In this context it must be noted that several genes of the type III secretion pathway of *Yersinia* spp., in particular LcrV, are down regulated and secretion and production of effector proteins is completely blocked in the presence of millimolar amounts of Ca^{2+} (Forsberg et al., 1987). It also became apparent from tissue culture infection models that the absence of Ca^{2+} in vitro mimics a yet undefined signal that is received by *Yersinia* species when they are adherent to eukaryotic cells and that induce both type III secretion genes and effector molecules such as YopE and Yops (Cornelis, 1998).

The dependence of *aexT* expression on type III secretion mechanism was also indicated by the presence of a consensus sequence upstream the *aexT* toxin gene in *A. salmonicida*, which shows full homology to the binding site of a transcriptional activator, known in *P. aeruginosa* as ExsA, which is involved in type III dependent gene expression (Frank, 1997). The expression of *aexT* in *A. salmonicida* is thus dependent on a functional type III secretion mechanism. The lack of production of AexT as detected in the type strain of *A. salmonicida* ATCC 33658^T as well as in the strain JF2397 which was derived from an originally virulent *A. salmonicida* strain, JF2267, in spite of the presence of a functional *aexT* gene, must therefore be due to the loss of the type III secretion pathway.

The AcrV protein of the novel type III secretion pathway of *A. salmonicida* plays an important role in pathogenesis by its role as a sensor and regulator of the system, as shown in other type III secretion systems. An important role in the secretion-related regulatory role in the

low Ca^{2+} response of *Y. pestis* is attributed to LcrV, which is localized to the bacterial surface and required for targeting of Yops of *Y. pestis* (Fields and Straley, 1999; Nilles et al., 1997). In addition, it was postulated that LcrV is also secreted by a special pathway which results its localization in the cytosol of infected cells but not the surrounding medium (Fields and Straley, 1999). Using a tissue cell model, it was shown that antiserum directed against LcrV prevented *Y. pestis* from injecting the Yop effector molecules into the host cells (Pettersson et al., 1999; Hueck, 1998). Active immunization of mice with recombinant LcrV antigen efficiently protected mice against challenge with *Y. pestis* (Leary et al., 1995). Our results showed that antibodies directed against recombinant AcrV, the analogous protein to LcrV, protected fish RTG-2 cells from damage caused by virulent *A. salmonicida* strain JF2267 and demonstrated that the AcrV plays an important role in type III secretion pathway mediated virulence of *A. salmonicida*.

The newly found type III secretion pathway plays a central role in pathogenicity of *A. salmonicida* via the secretion and direct injection of the ADP-ribosylating toxin AexT into the target cells. Loss of the type III secretion pathway, which is frequently observed, is due to the instability of a kb plasmid under culture conditions. Furthermore, loss of type III secretion genes such as *acrD* and *acrV* abolished expression of the *aexT* gene, and led to loss of virulence of *A. salmonicida*. As shown, surface exposed gene products of this type III secretion pathway, in particular AcrV, are potent candidates for new vaccines for the immune prophylaxis of fish against furunculosis.

The invention is further described by way of the following examples and results, which are not to be considered as limiting the scope of the invention. It will be appreciated by those skilled in the art, in light of this disclosure, that many changes can be made in the specific embodiments disclosed without departing from the scope of the invention.

EXAMPLES AND RESULTS

Materials and Methods

Bacterial strains, growth conditions and cloning vectors:

A. salmonicida strains are listed in Table 1. *A. salmonicida* type strain ATCC 33658^T was purchased from the American Type Culture Collection. *A. salmonicida* strain JF2267 was freshly isolated from an arctic char (*Savelinus alpinus*) showing typical symptoms of furunculosis. *A. salmonicida* strain JF2397 was derived from strain JF2267 by repeated single colony isolations after each of nine passages propagated on LB agar medium at 22°C for two days each passage.

A. salmonicida strains were routinely cultured on blood agar plates (Trypticase soy agar supplemented with 0.1% $CaCl_2$ and 5% sheep blood) at 19°C unless otherwise mentioned.

Liquid cultures of *A. salmonicida* were made by inoculation of Tripticase soy broth (TSB) (2.75 g/100 ml Trypticase soy broth without Dextrose (BBL® 11774, Becton Dickinson AG, Basle, Switzerland), 0.1% Glycerol, 0.1 M L-Glutamic acid pH 7.3) with fresh culture from solid medium and subsequent growth for 18 h at 19°C. For growth in Ca^{2+}-restricted medium, TSB was supplemented with 10 mM Nitrilotriacetic acid (Titriplex I, Merck 1.08416, Darmstadt, Germany).

For cloning and expression of cloned genes, *Escherichia coli* strains XL1-blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)] (Bullock et al., 1987), and BL21(DE3) (F'*dcm ompT hsdS*(r_B- m_B-) gal λ(DE3)) (Studier et al., 1990) respectively, were used. Plasmid pBluescriptII-SK$^-$ (Stratagene, La Jolla, CA, USA) was used as basic cloning vector. For the construction of genes encoding poly-Histidine fusion proteins and their expression, plasmid pETHIS-1, a T7 promoter based expression vector (Schaller et al., 1999) was used. *E. coli* strains were grown at 37°C in Luria-Bertani broth (LB) supplemented when necessary with ampicillin (50 μg/ml) for selection and maintenance of recombinant plasmids. When blue-white selection with pBluescriptIISK$^-$ was performed, 125μM X-Gal medium was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-thiogalacto-pyranoside.

Preparation of genomic DNA, cloning and sequencing procedures:

Genomic DNA of *A. salmonicida* was extracted by the guanidium hydrochloride method (Pitcher et al., 1989). A partial gene library of *A. salmonicida* JF2267 was constructed by cloning agarose gel purified *Sac*I-*Sal*I digested fragments of 4 to 6 kb size into vector pBluescriptII-SK$^-$ using standard procedures (Ausubel et al., 1999). Recombinant plasmids were screened by colony blot (Ausubel et al., 1999) using digoxigenin (DIG)-labeled DNA probes as described previously (Braun et al., 1999). Plasmids from *A. salmonicida* were purified using the method of Birnboim and Doly (Birnboim and Doly, 1979).

To construct a genomic library from *A. salmonicida* JF2267, 0.1 μg of DNA partially digested with *Sau*3a was ligated to ZapExpress *Bam*HI prepared arms (Pharmacia, Uppsala, Sweden) and packed into phage Lambda. Two-hundredμl of freshly grown XL1-blue MRF' cells (Pharmacia) resuspended in 10 mM $MgSO_4$ were infected with the packed phages during 15 min at 37°C. Three ml of preheated (50°C) Top Agarose (LB-broth containing 0.7% Agarose) supplemented with IPTG and X-Gal for blue/white selection were added and the mixture was poured onto an LB-Agar plate. Plates were incubated overnight at 37°C and then used for

screening of plaques. Positive plaques were cut out and stored overnight at 4°C in 0.5 ml SM-buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris, pH 7.5, and 0.01% gelatine) containing 20μl chloroform. 20 ml overnight cultures of XL1-blue MRF' grown in LB supplemented with 0.2% maltose and 10 mM $MgSO_4$ and 20 ml XLOLR cells (Pharmacia) grown in LB media were centrifuged for 5 min at 4'000 rpm and resuspended in 10 mM $MgSO_4$ to a final OD_{600} = 1. Two-hundredμl of the XL1-blue MRF' cells were added to 250μl of the SM-buffer containing the positive phages and 1μl (10^7 pfu) ExAssist™ helper phage. This mixture was incubated 15 min at 37°C and 3 ml LB-broth were added and shaken another 3 hrs at 37°C. The cultures were then heated for 15 min at 70°C, centrifuged during 15 min at 5'700 rpm, 4°C, and the supernatant containing the pBK-CMV phagemid filamentous phage was decanted into fresh tubes. Two-hundred μl XLOLR cells were mixed with 100μl supernatant and incubated for 15 min at 37°C, 300μl LB-broth were added and the culture was incubated for another one hr at 37°C. Two-hundred μl of this culture were plated on LB-plates containing 50 mg/l kanamycin overnight at 37°C. Colonies were picked and mini-preps (using the QIAprep Spin Miniprep kit, Qiagen AG, Basle, Switzerland) performed for plasmid purification.

For sequencing, subclones of sequential DNA segments were generated with a double-stranded nested deletion kit (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden). Sequencing was done with the dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol using either T3 and T7 primers flanking the cloned inserts in pBluescriptII-SK$^-$ or customer-synthesized internal primers. All sequences were determined on both strands. Reaction products were analyzed on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Sequence data analyses:

Sequence alignment and editing were performed by using the software Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). Comparisons of DNA sequences and their deduced amino acid sequences with EMBL/GenBank and NBRF databases were performed using the programs BLASTN, BLASTX and BLASTP (Altschul et al., 1990). Potentially antigenic segments of AcrV were determined using the software *ProtScale* (http://www.expasy.ch/cgi-bin/protscale.pl) (Bairoch et al., 1995) and the software *Coils output* (htpp://www.ch.embnet.org/software/COILS_form.html) (Lupas et al., 1991). The molecular masses of the protein and its theoretical isoelectric pH (pI) were calculated by using ProtParam tool (http:/www.expasy.ch/tools/ protparam.html) (Gill and von Hippel, 1989). Transmembrane prediction of the protein were made by using Tmpred (http://www.ch.embnet.org/software /TMPRED form.html) (Hofmann and Stoffel, 1993).

PCR amplifications and preparations of DIG-labeled gene probes:

Template DNA was produced either by extraction of genomic DNA or by preparation of lysates from bacterial colonies. Lysates were obtained by resuspending five colonies of the corresponding bacterial cultures in 200 µl lysis buffer (100 mM Tris-HCl, pH 8.5, 0.05% Tween 20 (Merck), 0.24 mg/ml proteinase K (Roche Diagnostics, Rotkreuz, Switzerland) dissolved in pyrogen-free water, filtered through a 0.22 µm low protein binding membrane filter) followed by subsequent incubation for 60 min at 60°C and 15 min at 97°C. Lysates were then cooled on ice and used as PCR templates.

PCR amplifications were performed with either a PE9600 or PE2400 automated thermocycler with MicroAmp tubes (Applied Biosystems). The reaction was carried out in a 50 µl reaction mix (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50mM KCl, 0.005% Tween 20, 0.005% NP-40 detergent, 170 µM of each deoxinucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 0.25 µM of each primer, 2.5 units *Taq* DNA polymerase (Roche Diagnostics)), and 100 ng of template DNA or 5 µl lysate. For the production of DIG-labeled probes, PCR mixtures were supplemented with 40 µM digoxigenin-11-dUTP (Roche Diagnostics). PCR conditions were as follows: 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 1 min at the corresponding annealing temperature (Table 2), and 30 s at 72°C. In addition, an extension step of 7 min at 72°C was added at the end of the last cycle in order to ensure full length synthesis of the fragments.

Curing of type III secretion genes from *A. salmonicida*:

In order to study the segregation of the type III secretion genes in *A. salmonicida* strain JF2267, the strain was inoculated in LB-broth at a density of A_{600} = 0.08 and incubated 2 ½ hrs at 19°C. Then the culture was split in two. One part was kept for continued growth at 19°C, while the other part was incubated at 22°C. Samples were taken at different time points from both cultures and spread on LB-agar medium. The plates were then incubated at 19°C for 24 hrs. Subsequently, colony blot hybridizations were performed using gene probes to determine the loss of specific genes.

Pulsed-field gel electrophoresis (PFGE):

The bacterial strains *A. salmonicida* JF 2267 and JF2397 were grown on LB agar for one day at room temperature. Then bacterial suspensions in 10 mM Tris, 10 mM EDTA, pH 8.0, sterile, were prepared to a final OD_{600} of 5. Three-hundred µl of 1.5% Sea Kem gold agarose (FMC Bioproducts, Maine, USA) in 100 mM Tris, 100 mM EDTA, pH 8.0, was added to 300µl

of bacterial cell suspension. Plugs were immediately poured in sterile moulds and kept on ice until hardened. The plugs were then incubated at 50°C overnight in sterile 1.5 ml 0.5 M EDTA, 1% N-lauroylsarcosin, 2 mg/ml proteinase K (Roche Diagnostics), pH 8.0, by shaking. The next day, the plugs were thoroughly washed 5 times over the whole day at room temperature in sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored in sterile 0.5 M EDTA, pH 8.0, at 4°C until further use. To digest the plugs they were first incubated in 4 x Buffer H (Roche Diagnostics) for 10 min at 22°C. Then the plugs were incubated at 37°C by shaking for 7 ½ hrs in 2 x Buffer H containing 40 U of *Not*I (Roche Diagnostics). They were then placed into the slots of a 1% Sea Kem gold agarose gel in 0.5 x TBE and sealed with 1% Sea Kem gold agarose. The gel was then equilibrated in 0.5 x TBE at 12°C using an Electrophoresis CHEF-DR® III system (BioRad Laboratories, Hercules, CA, USA). To separate *Not*I DNA fragments, the field was 6V/cm, having an angle of 120°, starting with 1 s and ending with 12 s. The duration of the PFGE was 14 hrs and it was performed at 12°C. The gel was stained 30 min at room temperature in water containing 0.5 μg/ml ethidium bromide, washed two times with water and analyzed under a UV-light. Additionally, the gel was further used for Southern-blotting.

Southern-blot analyses:

Southern-blotting was done by alkaline transfer onto positively charged nylon membranes (Roche Diagnostics) with an LKB 2016 VacuGene vacuum blotting pump (Pharmacia LKB). To depurinate the agarose gels they were incubated for 10 min in 0.25 M HCl, and subsequent transfer was performed with 0.4 M NaOH for 1 ½ hrs. After blotting, membranes were baked for 30 min at 80°C under vacuum. After at least one hr of prehybridization, hybridization was carried out in 5 x SSC (1x SSC in 0.15 M NaCl plus 0.015 M sodium citrate)-1% blocking reagent (Roche Diagnostics)-0.1% N-lauroylsarcosine sodium salt-0.02% sodium dodecyl sulphate (SDS) at 68°C overnight, using DIG-labeled DNA as probe. Membranes were washed under nonstringent conditions twice for 5 min each with 50 ml of 2x SSC-0.1% SDS per 100 cm^2 at 22°C, followed by medium-high-stringency washing twice for 15 min each with 50 ml of 0.2x SSC-0.1% SDS per 100 cm^2 at 68°C. The membranes were then processed with phosphatase-labeled anti-DIG antibody (Roche Diagnostics) according to the manufacturer's protocol. Signals were produced with chemiluminescent substrate (CSPD, Roche Diagnostics).

Pulsed-field gels were treated for Southern-blotting by using the same solutions as described above. To depurinate the agarose gels efficiently, they were incubated for 20 min in 0.25 M HCl, and then equilibrated for 20 min in 0.4 M NaOH. Transfer was performed for 3 hrs and the gels were treated as described above.

Expression and purification of His-tailed fusion protein AcrV:

Oligonucleotide primers used to amplify the whole *acrV* gene are given in Table 2. The PCR reactions were carried out as described above with the exception of using *Pwo* DNA polymerase (Expand Long Template PCR System kit, Roche Diagnostics) instead of *Taq* DNA polymerase and genomic DNA of *A. salmonicida* JF2267. The PCR products were purified by using the High Pure™ PCR Product Purification Kit (Roche Diagnostics) as described by the manufacturer's protocol. Then the *acrV* PCR product was cloned into pGEM-T vector (Promega, Madison, WI, USA), having 3'-T overhangs at the insertion sites, as described in the manufacturer's protocol and transformed into *E. coli* strains XL-1 Blue. The resulting plasmid was designated pJFFIVB873. The cloning of the PCR products into pGEM-T vector was used to provide efficient restriction of the subcloned fragments. Plasmid pJFFIVB873 was then digested with *Eco*RI and *Not*I, and the DNA fragment was inserted into the T7-promoter-based expression vector pETHIS-1 (Schaller et al., 1999). The resulting plasmid, pJFFETHISacrV4 was purified and controlled by DNA sequencing to assure the fusions with the vector's poly-His codons and then transformed into *Escherichia coli* BL21(DE3) cells (Novagen) for expression. Expression was induced by addition of 1 mM IPTG to cultures and incubation continued for another 3 h. The cells were sedimented by centrifugation at 3000 x g for 10 min, resuspended in 5 ml PN buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl), sonicated with a microtip for 4 min with the power output control at 1 and a duty cycle of 50% (1 s pulses) in a Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT, USA). Then guanidine hydrochloride was added to a final concentration of 6 M and was incubated overnight at 4°C on a shaker. The mixture was loaded onto a prewashed 2.5 ml bed volume Ni^{2+} chelation chromatography column (Qiagen) and washed once more with 30 ml PNG buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 6 M guanidine hydrochloride). Step elutions of the proteins were performed by adding 10 ml PNG buffer at each different pH (7.0, 6.0, 5.5, 5.0, and 4.5) and fractions of 1 ml were collected. The fractions were dialyzed and analyzed on 15% PAGE. The purified fusion proteins were eluted at pH 4.5.

Production of monospecific rabbit anti-AcrV antibodies and immunoblot analyses:

Monospecific, polyclonal antibodies directed against AcrV were obtained by immunizing rabbits subcutaneous with 80 µg of recombinant polyhistidine-tailed AcrV protein in 200 µl PN buffer and 150 µl NaCl (0.85%) mixed with 350 µl Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA) followed by a booster immunization with the same amount of protein in Freund's incomplete adjuvant (Difco) 3 weeks later. The animals were bled 22 d after the booster immunization according to standard protocols (Harlow and Lane, 1988).

Infection of fish cell cultures with *A. salmonicida*:

Rainbow trout (Oncorhynchus mykiss) gonad cells (RTG-2, ATCC CCL-55) were grown in 75 cm^2 tissue culture flasks (Techno plastic products AG, Trasadingen, Switzerland) at 22°C in minimum essential medium (GibcoBRL Life Technologies, Basel, Switzerland) supplemented with 2 mM L-glutamine (GibcoBRL), 1 x non-essential amino acids (GibcoBRL), 3 g/l sodium bicarbonate and 10% foetal bovine serum. Three days before infection the cells were trypsinized and 4 mio cells were seeded into a 25 cm^2 tissue culture flask. Monolayered RTG-2 cells were infected with *A. salmonicida* cells resuspended in phosphate buffered saline (PBS) pH 7.4 at a multiplicity of infection of 20:1 or 2:1 (bacteria/fish cells). As a control also 100 µl of pure PBS pH 7.4 were added to cultured fish cells. After 24 hrs of infection at 15°C the fish cells were photographed under a green filtered phase contrast microscope (Aixovert 100, Zeiss, Jena, Germany). To detach the cultured cells from the flask, the flask was shaken by hand. The suspended cells were centrifuged for 5 min at 4'000 rpm. Lysis of the fish cells was performed in 100 µl distilled water with two subsequent freeze thawing steps and verified by microscopy. The lysed fish cells were used for further analyzes on Western-blots.

Protection assay using rabbit antiserum AcrV:

RTG-2 fish cells were grown as described above. Two days before infection 20 mio of trypsinized RTG-2 fish cells were seeded into 24 well culture plates (1.9 cm^2) (Techno plastic products AG, Trasadingen, Switzerland). Rabbit antiserum directed against AcrV as well as control preserum were decomplemented for 30 min at 56°C. A fresh culture of *A. salmonicida* (at end exponential growth phase) was washed and resuspended in PBS pH 7.4 and mixed with either preserum or anti AcrV antiserum at a ratio of 1:1, 1:10, 1:100, 1:1000 or 1:10'000. Bacteria were incubated with the serum at 18°C for 30 min. The opsonized bacteria were added to the fish cells in a ratio of 20:1 or 2:1 (bacteria/fish cells). After 21 hrs of infection at 15°C the fish cells were photographed as described before and inspected for morphological changes.

SDS-PAGE and immunoblot analysis:

Proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (Laemmli, 1970) using 15 % or 10 % polyacrid gels and transferred to a nitrocellulose membrane (BioRad Laboratories). For immunoblotting, Western-blots were blocked with 1% milk buffer for at least one hour and then incubated with the rabbit antiserum AcrV (1:2000) or with the rabbit preserum (1:1000) in milk buffer overnight at 4°C. The membranes were then washed thoroughly with water before phosphatase-labelled conjugate (Goat anti-Rabbit IgG (H+L) [cat. no. 075-1506], Kirkegaard & Perry, Gaithersburg, MD, USA) diluted 1:2000 in milk buffer was added. The reaction was visualized 90 min later by incubation with BCIP-NBT (Ausubel et al., 1999).

Cloning and sequence analysis of the *virA* locus of a type III pathway of *A. salmonicida*:

Analysis of *A. salmonicida* strain JF2267 with an array of broad range probes for detection of type III secretion pathways revealed a strong signal with the *lcrD* subset of the probes, indicating the presence of a new type III secretion pathway. Subsequent Southern-blot analyses showed a 4.8 kb fragment of *Sac*I-*Sal*I digested genomic DNA of strain JF2267 reacting with the *lcrD* probe. This fragment was cloned on vector pBluescriptII-SK$^-$ leading to plasmid pJFFIVB638 which was subsequently sequenced. DNA sequence analyses revealed the presence of eight open reading frames (ORF) (figure 1) which showed strong similarity to the genes encoded on the *virA* operon of the type III secretion pathway of *Yersinia pestis* and *Pseudomonas aeruginosa*. In analogy to the *Y. pestis* genes, we named them *acr1*, *acr2*, *acr3*, *acr4*, and *acrD* (Aeromonas calcium response (Fig. 1)). They are located on a single operon followed by a transcription termination signal similar to the *virA* operon of *Y. pestis, Y. enterocolitica* and *Pseudomonas aeruginosa* (Boland et al., 1996; Iriarte and Cornelis, 1999; Plano et al., 1991; Cornelis, 1998; Yahr et al., 1997a). The similarities of the genes *acr1, acr2, acr3, acr4 and acrD* with the analogues in *Y. enterocolitica* and *in P. aeruginosa* are given in Table 2. Downstream *lcrD* we identified a locus with a canonical promoter sequence followed by further genes named *acrR*, *acrG*, and *acrV* on a separate operon (Fig. 1) according to the corresponding genes in *Y. pestis* (Table 3) (Barve and Straley, 1990; Skrzypek and Straley, 1993; Nilles et al., 1998). The ORF of the putative *acrV* gene seemed to be incomplete on the 4.8 kb SacI-SalI fragment of pJFFIVB638, and represented only the 5'-half of the gene. The remaining part of *acrV* and part of *acrH* located downstream of *acrV* were cloned separately from the λ phage gene library of *A. salmonicida* as an overlapping clone which was obtained by screening the gene library using a gene probe for the 5'-half of *acrV* which was produced by PCR with primers AcrV-L and AcrV-R (Table 2). The resulting plasmid based on vector pBK-CMV was designated pJFFIVB832. From this plasmid, a 0.9 kb *Sal*I fragment containing the 3' end of *acrV* and part of the downstream gene *acrH* was subcloned on pBluescriptII-SK and designated pJFFIVB828.

Instability of the genes belonging to the type III pathway in *A. salmonicida*:

When we analyzed the different *A. salmonicida* strains with a specific probe for *acrD*, we discovered by using Southern blot hybridization that the *acrD* gene was present only in strain JF2267 but not in the derivative strain JF2397 which had undergone nine passages of subsequent single colony cloning isolation. Additionally, the type strain of *A. salmonicida*, ATCC 33658^T, did not show a signal with the *acrD* probe. However, several *A. salmonicida* strains that were freshly isolated from salmon and trout with furunculosis did contain *acrD* (Table 4). These

results indicate that the type III secretion pathway of *A. salmonicida* may be lost easily. In order to get an estimate on the loss of the type III secretion genes, we have analyzed the kinetics of disappearance of *acrD* after a shift of growth temperature of strain JF2267 from 19°C to 22°C. Colony hybridization with the *acrV* probe revealed that in a fresh culture of strain JF2267, the *acrD* gene was present in all cells grown at 19°C. After the shift to 22°C, *acrD* was still present for further 5 ½ hrs, following which it was lost very rapidly within less than 1 hr (Fig. 2). Taking into account the generation time of 2 h for *A. salmonicida* under the given growth conditions, the *acrD* gene was lost within two generations. To analyze the loss of *acrD* further, undigested and NotI digested genomic DNA of *A. salmonicida* strain JF2267 and of the *acrD* deficient derivative strain JF2397 were submitted to pulse field gel electrophoresis (PFGE) and subsequent Southern blot hybridization with the *acrD* probe. PFGE analyses of total undigested DNA revealed the presence of two large plasmids in strain JF2267 while in strain JF2397 only one of the two plasmids was seen (Fig. 3). Digestion of the total DNA from these two strains with the rarely cutting enzyme *Not*I revealed the lack of a 84 kb band in strain JF2397 compared to JF2267 as the sole detectable difference (Fig. 3). Southern-blot hybridization of the DNA on this gels with the *acrD* probe confirmed the larger plasmid and the 84 kb *Not*I fragment of strain JF 2267 to contain *acrD* gene. Neither the remaining large plasmid in JF2397 nor any of its *Not*I fragments hybridized with the *AcrV* probe. This indicates that the type III secretion genes, or at least the *virA* operon thereof, are located on a large plasmid in the size range of 84 kb.

Presence of *acrD* in *A. salmonicida* strains:

In order to assess the presence of the *acrD* gene in various *A. salmonicida* strains, DNA samples extracted from *A. salmonicida* Type strain ATCC33658 and various field strains isolated from salmon or char were digested with restriction enzymes *Sal*I and *Sac*I, separated by 0.7% agarose gel electrophoresis, blotted onto nylon membranes and hybridized with the *acrD* gene probe. The Southern blot revealed the presense of the *acrD* gene on a 4.8 kb fragment in all strains except in the type strain ATCC33658, the laboratory strain JF2396 which was used for the type III secretion genes, and *A. salmonicida* strain MT44 known to be avirulent for trout. One field strain, # 24, showed a very weak hybridization signal indicating that the culture contains *acrD* only in a minor population of the cells (Table 1).

Infection of RTG-2 fish cells and protection of cell damage with anti-AcrV antiserum:

Freshly cultured *A. salmonicida* strain JF2267 was used to infect RTG-2 cells. After 24 hrs of incubation the fish cells were rounded up and also detached from the plastic support (figure 4A). In contrast, cells infected with *A. salmonicida* type strain ATCC 33658^T or strain JF2397 (figure 4B and C), both known to be devoid of *acrD* and *acrV*, showed no morphological

changes at all in spite of a massive multiplication of the bacteria in the cultures. RTG-2 fish cells which were incubated with PBS buffer as control showed no morphological changes like the cells infected with the *acrD* and *acrV* deficient strains JF2397 or ATCC 33658^T (figure 4D).

In order to study further the role of the newly detected type III secretion pathway in virulence of *A. salmonicida*, we incubated strain JF 2267 with monospecific polyclonal anti-AcrV antibodies prior to infection of RTG-2 fish cell cultures. When RTG-2 fish cells were infected with strain JF2267 that was incubated with rabbit anti-AcrV antibodies diluted 1:1 or 1:10, the characteristic morphological changes of the cells were reduced, significantly affecting only 20 % of the cells or less (Fig. 4E) compared to the infection with non-treated strain JF 2267 (Fig. 4A) or to the infection with JF 2267 that was pretreated with serum from the same rabbit taken before immunization (Fig. 4F). Titration of the anti-AcrV serum showed that protection of about 50% of the RTG-2 cells could still be reached with a serum dilution of 1:100, while further dilutions had no visible effect in protection.

Expression of AcrV in *A. salmonicida*:

The expression of AcrV in *A. salmonicida* strain JF2267 was assessed by immunoblots using AcrV-His antibodies. When *A. salmonicida* was grown under standard culture conditions in TSB medium, no AcrV protein could be detected from total cells nor from culture supernatant of strain JF 2267, nor in the control of strains JF2397 and ATCC33658^T. However, when the cells are submitted to a low Ca^{2+} response by chelating free Ca^{2+} ions in the growth medium by the addition of 10 mM NTA, we detected AcrV with anti-AcrV antibodies in the pellet of JF2267 as a protein of about 37 kDa (Fig. 5) but not in strains JF2397 and ATCC33658^T ,which are both devoid of the *AcrV* gene (Fig. 5). No AcrV protein could be detected in the supernatants of cultuires from strains JF2267, JF2396 and ATCC33658^T, grown in Ca^{2+} depleted medium.

When strain JF2267 was grown under standard culture conditions (containing free Ca^{2+} ions) and then put in contact with RTG-2 cells at a ratio 2:1 (bacteria : cells) for 30 minutes, the AcrV protein could be monitored on immunoblots reacting with anti-AcrV, similar to cultures from Ca^{2+} depleted medium.

Recombinant AcrV Vaccine Trial

(see Appendix A)

While particular elements, embodiments and applications of the present invention have been shown and described, it will be understood, of course, that the invention is not limited thereto, since modifications may be made by those skilled in the applicable technologies,

particularly in light of the foregoing description. The appended claims include within their ambit such modifications and variants of the exemplary embodiments of the invention described herein as would be apparent to those skilled in the applicable technologies

REFERENCES

Altschul,S.F., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J.: Basic local alignment search tool. J.Mol.Biol. 215 (1990) 403-410.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K.: Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y., 1999.

Bairoch,A., Bucher,P. and Hofmann,K.: The PROSITE database, its status in 1995. Nucleic Acids Res. 24 (1995) 189-196.

Barve,S.S. and Straley,S.C.: lcrR, a low-Ca^{2+} response locus with dual Ca^{2+} dependent functions in *Yersinia pestis*. J.Bacteriol. 172 (1990) 4661-4671.

Bergman,T., Hakansson,S., Forsberg,A., Norlander,L., Macellaro,A., Backman,A., Bolin,I. and Wolf-Watz,H.: Analysis of the V antigen *lcrGVH-yopBD* operon of *Yersinia pseudotuberculosis*: evidence for a regulatory role of LcrH and LcrV. J.Bacteriol. 173 (1991) 1607-1616.

Birnboim,H.C. and Doly,J.: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7 (1979) 1513-1523.

Boland,A., Sory,M.P., Iriarte,M., Kerbourch,C., Wattiau,P. and Cornelis,G.R.: Status of YopM and YopN in the Yersinia Yop virulon: YopM of *Y.enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus. EMBO J. 15 (1996) 5191-5201.

Braun,M., Kuhnert,P., Nicolet,J., Burnens,A.P. and Frey,J.: Cloning and characterization of two bistructural S-layer-RTX proteins from *Campylobacter rectus*. J.Bacteriol. 181 (1999) 2501-2506.

Bullock,W.O., Fernandez,J.M. and Short,J.M.: XL1-Blue: A high frequency efficiency plasmid transforming recA Escherichia coli strain with beta-galactosidase selection. Biotechniques 5 (1987) 376-378.

Cheng,L.W. and Schneewind,O.: *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for specific targeting of YopE, YopH, YopM, and YopN into the cytosol of eukaryotic cells. J.Bacteriol. 182 (2000) 3183-3190.

Chu,S., Cavaignac,S., Feutrier,J., Phipps,B.M., Kostrzynska,M., Kay,W.W. and Trust,T.J.: Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. J.Biol.Chem. 266 (1991) 15258-15265.

Cornelis,G.R.: The Yersinia Yop virulon, a bacterial system to subvert cells of the primary host defense. Folia Microbiol.(Praha) 43 (1998) 253-261.

Ellis,A.E.: Immunization with bacterial antigens: furunculosis. Dev.Biol.Stand. 90 (1997) 107-116.

Fenselau,S., Balbo,I. and Bonas,U.: Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. Mol.Plant Microbe Interact. 5 (1992) 390-396.

Fields,K.A. and Straley,S.C.: LcrV of *Yersinia pestis* enters infected eukaryotic cells by a virulence plasmid-independent mechanism. Infect.Immun. 67 (1999) 4801-4813.

Forsberg,A., Bolin,I., Norlander,L. and Wolf-Watz,H.: Molecular cloning and expression of calcium-regulated, plasmid-coded proteins of *Y. pseudotuberculosis*. Microb.Pathog. 2 (1987) 123-137.

Frank,D.W.: The exoenzyme S regulon of *Pseudomonas aeruginosa*. Mol.Microbiol. 26 (1997) 621-629.

Gill,S.C. and von Hippel,P.H.: Calculation of protein extinction coefficients from amino acid sequence data [published erratum appears in Anal Biochem 1990 Sep;189(2):283]. Anal.Biochem. 182 (1989) 319-326.

Gough,C.L., Genin,S., Zischek,C. and Boucher,C.A.: *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol.Plant Microbe Interact. 5 (1992) 384-389.

Harlow,E. and Lane,D.: Antibodies. A laboratory manual. Cold Spring Harbor Laboratory, 1988.

Hirono,I. and Aoki,T.: Cloning and characterization of three hemolysin genes from *Aeromonas salmonicida*. Microb.Pathog. 15 (1993) 269-282.

Hofmann,K. and Stoffel,W.: TMbase - A database of membrane spanning proteins segments. Biol.Chem.Hoppe-Seyler 347 (1993) 166

Hueck,C.J.: Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol.Mol.Biol.Rev. 62 (1998) 379-433.

Iriarte,M. and Cornelis,G.R.: Identification of SycN, YscX, and YscY, three new elements of the *Yersinia* yop virulon. J.Bacteriol. 181 (1999) 675-680.

Laemmli,U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680-685.

Leary,S.E., Williamson,E.D., Griffin,K.F., Russell,P., Eley,S.M. and Titball,R.W.: Active immunization with recombinant V antigen from *Yersinia pestis* protects mice against plague. Infect.Immun. 63 (1995) 2854-2858.

Lee,K.K. and Ellis,A.E.: Glycerophospholipid:cholesterol acyltransferase complexed with lipopolysaccharide (LPS) is a major lethal exotoxin and cytolysin of *Aeromonas salmonicida*: LPS stabilizes and enhances toxicity of the enzyme. J.Bacteriol. 172 (1990) 5382-5393.

Lupas,A., Van,D.M. and Stock,J.: Predicting coiled coils from protein sequences. Science 252 (1991) 1162-1164.

Michiels,T. and Cornelis,G.R.: Secretion of hybrid proteins by the *Yersinia* Yop export system. J.Bacteriol. 173 (1991) 1677-1685.

Motin,V.L., Nakajima,R., Smirnov,G.B. and Brubaker,R.R.: Passive immunity to yersiniae mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide. Infect.Immun. 62 (1994) 4192-4201.

Munro,A.L. and Hastings,T.S.: Furunculosis. In Inglis,V., Roberts,R.J. and Bromage,N.R. (Eds.), Bacterial diseases of fish. Blackwell Scientific, Oxford, 1993, pp.122-142.

Nilles,M.L., Fields,K.A. and Straley,S.C.: The V antigen of *Yersinia pestis* regulates Yop vectorial targeting as well as Yop secretion through effects on YopB and LcrG. J.Bacteriol. 180 (1998) 3410-3420.

Nilles,M.L., Williams,A.W., Skrzypek,E. and Straley,S.C.: *Yersinia pestis* LcrV forms a stable complex with LcrG and may have a secretion-related regulatory role in the low-Ca^{2+} response. J.Bacteriol. 179 (1997) 1307-1316.

Pettersson,J., Holmstrom,A., Hill,J., Leary,S., Frithz-Lindsten,E., Von Euler-Matell,A., Carlsson,E., Titball,R., Forsberg,A. and Wolf-Watz,H.: The V-antigen of yersinia is surface exposed before target cell contact and involved in virulence protein translocation. Mol.Microbiol. 32 (1999) 961-976.

Pitcher,D.G., Saunders,N.A. and Owen,R.J.: Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett.Appl.Microbiol. 8 (1989) 151-156.

Plano,G.V., Barve,S.S. and Straley,S.C.: LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. J.Bacteriol. 173 (1991) 7293-7303.

Price,S.B. and Straley,S.C.: *lcrH*, a gene necessary for virulence of *Yersinia pestis* and for the normal response of *Y. pestis* to ATP and calcium. Infect.Immun. 57 (1989) 1491-1498.

Sawa,T., Yahr,T.L., Ohara,M., Kurahashi,K., Gropper,M.A., Wiener-Kronish,J.P. and Frank,D.W.: Active and passive immunization with the Pseudomonas V antigen protects against type III intoxication and lung injury [see comments]. Nat.Med 5 (1999) 392-398.

Schaller,A., Kuhn,R., Kuhnert,P., Nicolet,J., Anderson,T.J., MacInnes,J.I., Segers,R.P.A.M. and Frey,J.: Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology 145 (1999) 2105-2116.

Skrzypek,E. and Straley,S.C.: LcrG, a secreted protein involved in negative regulation of the low-calcium response in *Yersinia pestis*. J.Bacteriol. 175 (1993) 3520-3528.

Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W.: Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185 (1990) 60-89.

Thornton,J.C., Garduno,R.A., Carlos,S.J. and Kay,W.W.: Novel antigens expressed by *Aeromonas salmonicida* grown in vivo. Infect.Immun. 61 (1993) 4582-4589.

Titball,R.W. and Munn,C.B.: The purification and some properties of H-lysin from *Aeromonas salmonicida*. J.Gen.Microbiol. 131 (1985) 1603-1609.

Whitby,P.W., Landon,M. and Coleman,G.: The cloning and nucleotide sequence of the serine protease gene (aspA) of *Aeromonas salmonicida* ssp. salmonicida. FEMS Microbiol.Lett. 78 (1992) 65-71.

Yahr,T.L., Goranson,J. and Frank,D.W.: Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. Mol.Microbiol. 22 (1996) 991-1003.

Yahr,T.L., Mende-Mueller,L.M., Friese,M.B. and Frank,D.W.: Identification of type III secreted products of the *Pseudomonas aeruginosa* exoenzyme S regulon. J.Bacteriol. 179 (1997b) 7165-7168.

Yahr,T.L., Mende-Mueller,L.M., Friese,M.B. and Frank,D.W.: Identification of type III secreted products of the Pseudomonas aeruginosa exoenzyme S regulon. J.Bacteriol. 179 (1997a) 7165-7168.

Appendix A

Recombinant AcrV Vaccine Trial

Materials:

VACCINE FORMULATIONS:

1. The **AcrV** vaccine was formulated using recombinant, Histidine-tagged AcrV resuspended in 10 mM phosphate buffer, pH 7.0, to 112.5 µg/mL. Four parts of this protein solution were mixed with one part oil adjuvant for a final AcrV concentration of 90 µg/mL. The dose for testing was 0.1 mL, or 9 µg/fish.
2. The commercial comparator vaccine was serial 4-13 of the vaccine **MultiVacc4** (Bayotek International Ltd.)
3. The placebo (control) vaccine consisted of phosphate buffered saline(**PBS**) (10 mM phosphate, 150 mM NaCl, pH 7.2).
4. All vaccines were maintained at 4°C until use.

Methods

TRIAL DESIGN:

Fish (rainbow trout *Oncorhynchus mykiss*) that have been determined to be pathogen free and are at least 15g in size are held for at least one-week pre vaccination for acclimation purposes. During the acclimation period the fish are offered 1% body weight in salmonid fish food every day, however they are denied food 24 hours pre and post-vaccination.

At least 50 fish are vaccinated 0.1 mL of **AcrV vaccine** via intra-peritoneal (IP) injection, or 0.2 mL of the commercial vaccine **MultiVacc4**. At the same time a group of at least 50 fish from the same stock are mock vaccinated with 0.1 mL of **PBS**. Vaccinated fish are then held for a period of at least 350-degree days to allow specific immune response generation in an acclimation tank with a continuous flow of water at a temperature of 12-13°C. The fish are offered 1% body weight in salmonid fish food daily until 24 hours pre- challenge and post-challenge.

After at least 350-dgree days post vaccination 50 fish per group were challenged by IP injection with a pre-determined concentration of virulent *Aeromonas salmonicida*. The dosage depends on the source of the fish and the water temperature (this is determined empirically immediately prior to challenge of test fish). The identical procedure is performed with the placebo vaccinated control fish. The fish are observed daily for mortality for 21 days post challenge and the cause of mortality assessed and examined to ensure that mortality is attributed to the challenge organism. After 24 hours post-challenge the fish are again offered 1% body weight in salmonid fish feed daily. Tanks are maintained with a continuous flow of water at a temperature of 12-13°C. For a challenge series to be considered satisfactory; all challenge groups must meet the following criteria:

1. At least 70% of the non-immunized controls must die within 21 days of challenge.

2. A relative percent survival (RPS) of no less than 25% must be achieved for the challenge disease before a vaccine is considered even partially efficacious for this disease.

RPS = [1-(% mortality vaccinates/% mortality controls)] x 100

Developed from: The Rules Governing Medicinal Products in the European Union, Volume VII, Guidelines for the testing of veterinary medicinal products. 1994. Specific Requirements for the Production and Control of Live and Inactivated Vaccines Intended for Fish. Section 3.2. Potency.

RESULTS

Group	% Mortality	RPS
PBS	82	-
AcrV	49	40
MultiVacc4	30	63

1. There was a strong challenge with 82% control mortalities.

Table 1

A. salmonicida strains used in this study and presence of *acrD*

strain	origin	*acrD* [a]
ATCC33658	American Type Culture Collection, Type strain	-
JF2267	Char (Savelinus alpinus), Switzerland	+
JF2396	Laboratory strain, derivative of JF2267	-
CC-23	Salmon, Norway	+
CC-24	Salmon, Norway	+/- [b]
CC-27	Salmon, Norway	+
CC-29	Salmon, Scotland, UK	+
CC-30	Salmon, Canada	+
CC-34	Salmon, Canada	+
MT 44	Spontaneous non virulent mutant	-
CC-63	Salmon, Canada	+
CC-72	Salmon, Canada	+

[a] as determined by Southern blot hybridization

[b] very weak hybridization signal indicating that only a minor part of the population of the culture contains the *acrD* gene

Table 2.

Oligonucleotide primers

Name	Sequence[a] 5' to 3'	Position[b]	Annealing temp. °C
AslcrD-L[c]	GCCCGTTTTGCCTATCAA	1159-1176	60
AslcrD-R[c]	GCGCCGATATCGGTACCC	2028-2011	60
AcrV-L[c]	TTCGTCGGCTGGCTTGATGT	4144-4163	58
AcrV-R[c]	GAACTCGCCCCCTTCCATAA	4734-4715	58
AsacrVt-L[d]	gggaattcGATGAGCACAATCCCTGACTAC	4104-4125	57
AsacrVt-R[d]	atgcggccgcAAATTGCGCCAAGAATGTCG	5188-5169	57
AsacrVN'-R[d]	tcgcggccgcACCCTTTACGCTGATTGTC	4555-4537	57
AsacrVC'-L[d]	cggaattcGTTGCGGGATGAGCTGGCAG	4554-4573	57
AsacrVC'-R[d]	tcgcggccgcACTCGGCTTCTATGCCACTC	4987-4968	57

[a] Lowercase letters indicate nucleotides added to create restriction enzyme recognition sites (underlined) for cloning.
[b] Based on nucleotide sequence of *A. salmonicida* JF2267
[c] Primer used for gene probe preparation
[d] Primer used for amplification of gene *acrV*, *acrV*-N, and *acrV*-C respectively

Table 3
A. salmonicida type III proteins compared to analogues in *P. aeruginosa* and in *Y. enterocolitica*.

Protein in *A. salmonicida*	Analogue in *P. aeruginosa*	Similarity / identity [a]	Genbank access. nr.	Analogue in *Y. enterocolitica*	Similarity / identity [a]	Genbank access. nr.	*Proposed function*
AcrI	PcrI	80 / 60	AF010150	TyeA	83 / 69	AF102990	part of the translocation-control apparatus, required for selective translocation of Yops
AcrZ	PcrZ	63 / 44	AF01050	SycN	77 / 62	AF102990	chaperone forYopN .
AcrH	PcrH	78 / 65	AF010149	LcrH (SycD)	79 / 58	AF102990	regulation of low calcium response, chaperon for YopD , secretion

[a] given as % of similar / identical amino acids

DNA sequence information

```
LOCUS       A.salmonicida type III vir1   5678 bp  DNA   BCT   26-MAR-2001
DEFINITION  Aeromonas salmonicida.
ACCESSION   tmpseq_1
VERSION
KEYWORDS    .
SOURCE      Aeromonas salmonicida.
  ORGANISM  Aeromonas salmonicida
            Bacteria; Proteobacteria; gamma subdivision; Aeromonas group;
            Aeromonas.
REFERENCE   1  (bases 1 to 5678)
  AUTHORS   Stuber,K. and Frey,J.
  TITLE     Detection of a novel type III secretion pathway in Aeromonas
            Salmonicida and its impact in virulence
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 5678)
  AUTHORS   Stuber,K. and Frey,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-MAR-2001) Institute for Veterinary Bacteriology,
            University of Berne, Laenggass-Strasse 122, Berne, BE 3008,
            Switzerland
FEATURES             Location/Qualifiers
```

SEQ ID. NO:1

```
     source          1..5678
                     /organism="Aeromonas salmonicida"
     gene            <1..144
                     /note="partial acr1 gene"
     CDS             1..144
                     /note="partial acr1 gene"
                     /codon_start=1
                     /transl_table=11
                     /product="partial Acr1"

/translation="ELKRLIRLLPVELFSEEEQRQNLLQCCQGALDNAIEREEDELSG
                     ESS"
```

SEQ ID. NO:2

```
     RBS             130..135
                     /note="acr2"
     gene            141..512
                     /note="acr2"
     CDS             141..512
                     /note="acr2"
                     /codon_start=1
                     /transl_table=11
                     /product="Acr2"

/translation="MNWIEPLLVQFCQDLGITIGDNPHSLIQLELEQSGTLQLERHQG

QLTLWLARAVPWHQSGEAIRRAMTLTAAAQGPALPVRSGWLGEEQLILFVSLDERAVT
                     LPQLHQAVTTLTRLQREVLAS"
```

SEQ ID. NO:3

```
     RBS             493..498
                     /note="acr3"
     gene            509..874
                     /note="acr3"
     CDS             509..874
                     /note="acr3"
                     /codon_start=1
                     /transl_table=11
                     /product="Acr3"

/translation="MSRITAAHIGIEQLSAISLDDQERSLPGRYALLPDGQSIEPHIS

RLYPERLADRVLLDFATPDRGFHDLLRPVDFNQAMQGLRSVLAEGQSPELRAAAALLE
                     QMHADEQLMQMTLHLLHKV"
```

SEQ ID. NO:4

```
     RBS             866..869
                     /note="acr4"
     gene            871..1221
                     /note="acr4"
     CDS             871..1221
                     /note="acr4"
                     /codon_start=1
                     /transl_table=11
                     /product="Acr4"

/translation="MTMVLTSQQQDALLLTGWLQLQYGHPDKASVLLAALLQIHPDHQ

GGRRTLLVALLKQGEGEAALAHVDQLMQQGEADGPLWLCRSRACQLAGRLDEARFAYQ
                     QYLELEEQNESTHP"
```

SEQ ID. NO:5

```
     RBS             1191..1197
                     /note="acrD"
     gene            1202..3319
                     /note="acrD"
     CDS             1202..3319
                     /note="acrD"
                     /codon_start=1
                     /transl_table=11
                     /product="AcrD"

/translation="MNQRTLELLRRIGERKDIMLAILLLAIVFMMVLPLPPVALDILI

AINMTISVVLLMMAVYINSPLQFSAFPAVLLITTLFRLALSVSTTRMILLQADAGQIV

YTFGNFVVGGNLVVGIVIFLIITIVQFLVITKGSERVAEVSARFSLDAMPGKQMSIDG

DMRAGVIDVHEARDRRGVIEKESQMFGSMDGAMKFVKGDAIAGLIIIFVNILGGVTIG

VTQKGLSAADALQLYSILTVGDGMVSQVPALLIAITAGIIVTRVSSEESSDLGTDIGA

QVVAQPKALLIGGLLLVLFGLIPGFPMITFFALSAIVTAGGYFIGLRQRKAQSSNSQD

LPAVLAQGAGAPAARSKPKPGSKPRGKLGEKEEFAMTVPLLIDVDAALQAELEAIALN

DELVRVRRALYLDLGVPFPGIHLRFNEGMGPGEYLIQLQEVPVARGLLRPGHQLVQES

ASQLDLLGIPYEEGAPLLPGQPTLWVANEHQERLEKSRLATLTTDQVMTWHLSHVLRE
```

YAEDFIGIQETRYLLEQMEGSYSELVKEAQRIIPLQRMTEILQRLVGEDISIRNMRAI

LEAMVEWGQKEKDVVQLTEYIRSSLKRYICYKYANGNNILPAYLLDQQVEEQLRGGIR

QTSAGSYLALDPTITQSFLDQVRHTVGDLAQMQNKPVLIVSMDIRRYVRKLIEGDYHA
LPVLSYQELTQQINIQPLGRVCL"

SEQ ID. NO:6

```
     stem_loop       3371..3412
                     /note="putative transcription stop signal"
     -35_signal      3416..3423
     -10_signal      3440..3447
     RBS             3476..3481
                     /note="acrR"
     gene            3490..3771
                     /note="acrR"
     CDS             3490..3771
                     /note="acrR"
                     /codon_start=1
                     /transl_table=11
                     /product="AcrR"

/translation="MLVRREGERAGLANPFAALYLLAEATLAVLGPGHFLYGNVDVFR
                     SSSLSSERLGRFYLRWTGASEPEPGWFMLATEQVCSLRDMRKRQKHGLA"
```

SEQ ID. NO:7

```
     RBS             3801..3895
                     /note="acrG"
     gene            3811..4095
                     /note="acrG"
     CDS             3811..4095
                     /note="acrG"
                     /codon_start=1
                     /transl_table=11
                     /product="AcrG"

/translation="MKQPRFADHSETISQAEHGIADSDHRNALLQEMLAGLALSDQTC
                     QLLFEAPTEQVAVAEQELLAEIQRRQALLPAQPGEGRKSRRPTIMRGLMI"
```

SEQ ID. NO:8

```
     RBS             4095..4099
                     /note="acrV"
     gene            4105..5190
                     /note="acrV"
     CDS             4105..5190
                     /note="acrV"
                     /codon_start=1
                     /transl_table=11
                     /product="AcrV"

/translation="MSTIPDYNTNPGAFVGWLDVQALNTLPGNKNPKLTELVELLKGK

ITISADSSTALSKEQLEKLLAAYLTDPASINGGWAMGQFKGGQDAAIAAIKGVIERGA

KQTPPVTHWTIPEFMLLSLSALTMERTDDDLITTFTGVMMFQDNQRKGLRDELAEMTA

ELKIYGVIQSEINQVLSAASNQTFKTNFNLMDYKLYGYESLAKFMEGGEFKLLSKMFS

DEQVTKAQQDFTNAKNELENVTSTSLNPKIQAEAKTDYERKKAIFEEIVETQIITLKT

FLESDLKKSGAMSGIEAEYKYDKDNNKLGNFSTSVSDRSRPLNDLVSEKTARLNDVSS
```

RYNAAIEALNRFIQKYDSIMRDILGAI"

SEQ ID. NO:9

```
     RBS             5189..5195
                     /note="acrH"
     gene            5200..5676
                     /note="partial acrH gene"
     CDS             5200..5676
                     /note="partial acrH gene"
                     /codon_start=1
                     /transl_table=11
                     /product="partial AcrH"

/translation="MQTDTTLTPEYEAELEAFMADGGTLAMLQDISGDTLEQLYALAF

SQYQAGKWEDAHKIFQALCMLDHYEPRYFLGLGACRQAMGEFETAVQSYSFGAMLDLK

DPRFPFHAGECRLQQGDLNGAESGFHSARLLADTDPQQADLAASAKVMLEAIAIRRD"
```

SEQ ID. NO:10

```
BASE COUNT     1172 a   1653 c   1731 g   1122 t
ORIGIN
       1 gagctcaagc ggctgatccg cctgctgccg gtggagctgt tcagtgaaga ggagcagcgc
      61 cagaatctgt tgcagtgctg tcagggtgcg ctcgataacg ccatcgagcg ggaagaggat
     121 gagttgtctg gagagtcgtc atgaactgga ttgaacccct gctggtgcag ttttgccagg
     181 atttgggcat caccataggg gataaccccc attcgctgat ccagcttgaa ctggagcaga
     241 gcggcactct gcagctggag cgccatcagg ggcaactgac cctatggttg gcccgcgccg
     301 tgccctggca tcagagtggc gaggccattc gccgcgccat gaccttgact gccgcggcgc
     361 aagggccggc actgccggtg cgcagcggct ggttgggggа ggagcagttg atcctcttcg
     421 tctccctgga tgagcgggcc gtgactctgc cccagctcca tcaggccgtg accaccctga
     481 cccggttgca gcgagaggtg ctggcgtcat gagccggatc actgccgcgc atatcggtat
     541 cgagcagctc agcgccatct ccctcgacga tcaggagcgc agcctgccgg ggcgttatgc
     601 cctgttgccc gatggccagt ccatcgaacc ccatatcagc cgcctctacc ccgagcggct
     661 ggcggatcgg gtgctgctcg atttcgccac cccggatcgc ggctttcacg acttgctgcg
     721 accggtcgat ttcaatcagg cgatgcaggg gctgcgcagt gtgctggcag aggggcagag
     781 ccccgaattg cgagcggccg ccgcgctgct cgaacaaatg cacgccgatg aacaactgat
     841 gcagatgacc cttcatctgc tgcacaaggt atgaccatgg tgcttacgtc acagcagcag
     901 gatgcgctgc tgctcaccgg ctggttgcaa ctgcaatatg gccaccctga caaggcgagc
     961 gtgctgctgg ccgccctgct gcagatccac cccgaccatc agggagggcg acggaccttg
    1021 ctggtggccc tgctcaaaca gggggagggg gaggcggcgc tggcccatgt cgatcagctg
    1081 atgcagcaag gggaggccga cggcccgctc tggctctgtc gcagccgagc ctgccagttg
    1141 gcagggcggc tggatgaagc ccgttttgcc tatcaacaat acctcgaact ggaagagcag
    1201 aatgaatcaa cgcacccttg agttgctgcg ccggataggc gaacgcaagg acatcatgct
    1261 ggcgatcctg ctgctggcca tcgtctttat gatggtcttg ccgctgccgc cggtggccct
    1321 cgatatcctg attgccatca acatgaccat ctcggtggta ctgctgatga tggcggttta
    1381 tatcaattcg ccgctgcagt tctccgcctt tccggcggtg ctgctgatca ccaccctgtt
    1441 ccggcttgcc ttgtcggtga gtaccacccg gatgatcctg ctgcaggctg atgcggggca
    1501 gatagtctac accttcggca acttcgtggt gggggggcaat ctggtggtgg ggatcgtcat
    1561 cttcctcatc atcaccatcg tccagtttct ggtgatcacc aagggctcgg agcgggtcgc
    1621 cgaggtgagc gcccgctttt ccctcgatgc catgccgggt aagcagatga gtatcgatgg
    1681 tgacatgcgc gccgggggtga tcgacgtgca cgaggcgcgg gatcgccgcg gggtcatcga
    1741 gaaggagagc cagatgttcg gctccatgga tggcgccatg aagtttgtga agggggacgc
    1801 catcgcgggc ctcatcatca tcttcgtcaa catcctcggt ggcgtcacca tcggggtgac
    1861 ccagaagggg ttatccgccg ccgatgcgct gcagctctac tccatcctga cggtgggtga
    1921 tggcatggtc tcccaggtgc cggcgctgct gatcgccatc accgcgggca ttatcgtcac
    1981 ccgggtctcc tccgaagagt cttccgatct gggtaccgat atcggcgccc aggtggtggc
    2041 ccagcccaag gcgctactga tcggcggtct gctgctggtg ctgttcgggt tgatcccggg
    2101 cttcccgatg atcaccttct ttgcgctgtc ggccatcgtc acggcgggcg gttactttat
    2161 cggcttgcga caacgcaagg cgcaaagcag caacagtcag gatcttcctg ccgtgctggc
    2221 gcaggggccc ggggccccag ctgcccgcag caagccaaaa ccgggcagca agccgcgggg
    2281 caagctgggg gagaaggagg agtttgccat gacggtgccg ctccttatcg atgtggatgc
    2341 tgctttgcag gccgagctgg aggcgattgc cctcaacgac gaactggtgc gggtgcgccg
    2401 cgccctctat ctcgatctcg gggtgccttt cccgggtatt cacctgcgtt tcaacgaggg
```

```
2461 gatggggcct ggcgaatacc tgatccagct gcaggaggtg ccggtcgccc gcggtctgct
2521 gcgcccgggc catcagctgg tgcaggagag cgcctcccag ctcgatctgc tggggatccc
2581 ctacgaagag ggggcgccgt tactgccggg acaaccgacc ttgtgggtcg ctaatgaaca
2641 tcaggagcga ctggagaagt cacggctggc caccctcacc accgatcagg tgatgacctg
2701 gcatctatcc catgtgctgc gggaatatgc cgaggacttt atcggcattc aggagacccg
2761 ctacctgctg gagcagatgg aggggagcta tagcgagctg gtgaaggagg cgcaacgcat
2821 catcccgctg cagcgtatga ccgaaatttt gcagcggctg gtgggggagg atatctccat
2881 ccgcaacatg cgcgccatcc tcgaggcgat ggtggagtgg ggccagaagg agaaggatgt
2941 ggtgcagctc accgagtaca tccgtagcag cctcaagcgc tacatctgct acaagtacgc
3001 caacggcaac aacatttgc ctgcctatct gctcgatcag caggtggagg agcagctccg
3061 cggcggcatt cgccagacta gtgccggcag ctatctggcg ctcgatccca ctattaccca
3121 gagcttcctc gatcaggtgc gccacaccgt cggtgatctg gcccagatgc agaacaaacc
3181 ggtgctcatt gtctccatgg atatccgccg ctatgtgcgc aagctcatcg agggggatta
3241 ccatgccctg ccggtgctct cctatcagga gctgacccag cagatcaata tccagccccc
3301 cgggagggtc tgcctgtgag gggggacccg ttaacctctg acccccctgat cccctggctg
3361 caggccaagg gtgtggcggt tgcctctcac tatctggggg caacccccat ccagctcggc
3421 cacgctttct gctatcgcca aatttatctc gcctggcggg ttgatcctac gacccgacgg
3481 gtctggatca tgctggtgcg ccgagagggg gagcgggctg gactggccaa tccctttgcc
3541 gccctctatc tgctggccga agccactctg gctgtactcg gtccgggcca tttcctctac
3601 ggcaacgtcg atgtctttcg aagcagtagc ctgagcagtg agcggctagg ccgcttctac
3661 ttgcgctgga cgggagccag tgaacccgag cccggctggt tcatgttggc caccgagcaa
3721 gtctgttcac tacgggatat gcgaaaacga caaaagcacg gccttgcgtg acaggcatgt
3781 ccaaaagggc ctcatagaat aggagccaag atgaaacaac cgcgttttgc cgaccatagc
3841 gagaccattt cgcaggcaga gcatggcatt gccgacagcg atcaccgcaa tgccctgttg
3901 caagagatgc tggctggcct agccctctcg gatcagacct gtcagctgct gttcgaagcg
3961 ccgaccgagc aagtggccgt ggccgagcag gagttgttgg cagagatcca gcgcagacag
4021 gcgttactac cggcacagcc gggagagggc cgcaaaagtc gccgtcccac cattatgcgc
4081 ggactgatga tttaaggagt cgtgatgagc acaatccctg actacaacac taaccccggc
4141 gcgttcgtcg gctggcttga tgtgcaagca ctgaacacat tgccgggcaa taaaaatccc
4201 aagttgaccg aactggtcga gctgctcaag ggcaagatca ccatcagtgc tgactcatcg
4261 actgcgctga gcaaggagca gctggagaag ttgctggctg cctatctgac ggatcctgcc
4321 tcgatcaacg ggggctgggc gatgggccag ttcaagggag gtcaagatgc cgccattgcc
4381 gccatcaagg gggtgatcga gcggggagca aaacaaaccc cgccagtcac ccactggacc
4441 atccctgaat ttatgctgct ctccctcagt gcgctgacca tggaacgtac cgatgacgat
4501 ctcatcacga cctttaccgg ggtgatgatg tttcaggaca atcagcgtaa agggttgcgg
4561 gatgagctgg cagagatgac cgctgagctg aagatctacg gggtgatcca gtccgagatc
4621 aaccaggtgc tctctgcggc gtccaaccaa accttcaaaa ccaatttcaa tctgatggat
4681 tacaagctct atggctatga gtctctggcc aaatttatgg aaggggggcga gttcaagctg
4741 ttgtcaaaaa tgtttagcga tgagcaggtg acaaaagcac agcaagattt caccaatgct
4801 aaaaatgagc tggaaaacgt cacgtcgacc agcctaaacc ccaaaatcca ggcggaagct
4861 aagaccgatt atgagcgtaa aaaagccatt tttgaggaga tcgtagagac gcagatcatc
4921 acccttaaaa cgttcctgga aagtgacctg aagaagagcg gcgccatgag tggcatagaa
4981 gccgagtaca aatatgacaa agacaacaac aagcttggca acttctccac tagtgtgagc
5041 gaccgttctc gcccgctcaa cgatctggtc agtgaaaaga ccgcccgcct caacgacgtc
5101 agttcgcgct acaacgctgc catcgaggca ctcaaccgct ttatccagaa atacgacagc
5161 atcatgcgcg acattcttgg cgcaatttga ggagagatca tgcagaccga caccaccctg
5221 accccggaat atgaagcaga gctggaggcc tttatggctg acggtggtac cctggctatg
5281 ctgcaggata tctctggcga caccttggaa cagctctatg ccctggcctt tagccagtat
5341 caggccggca agtgggaaga tgctcacaaa atcttccagg ctctctgcat gctggatcac
5401 tacgagccac gctatttcct cgggctgggt gcttgccgtc aggcgatggg ggagtttgaa
5461 acggcagttc agagttacag ctttggcgcc atgctcgacc tgaaagatcc ccgtttccca
5521 tttcatgcag gcgagtgccg gctgcaacaa ggtgatttga acggtgccga gagtggcttc
5581 cactcggccc gactgctggc ggacacagat ccccagcagg cagacctggc ggcaagcgcc
5641 aaggtcatgt tggaagccat cgcaatcaga agggatcc
```

//